Bioactive Peptides

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3.1 Introduction

Proteins and peptides from food are essential components of the diet as sources of amino acids. It has been also recognized that dietary proteins and peptides exert many other functionalities in vivo, directly or after hydrolysis. Several biologically active peptides have been identified after hydrolysis of food proteins by digestive enzymes during gastrointestinal transit or by fermentation or ripening during food processing. These bioactive peptides have been defined as specific amino acid sequences that may exhibit regulatory functions in the organism beyond normal and adequate nutrition. Once ingested, they may exert different activities in the major body systems: cardiovascular, digestive, endocrine, immune, and nervous system. Although any food protein from plant or animal origin may act as a precursor of these bioactive peptides, to date, most of the bioactive peptides described and commercially used derive from milk proteins. Research in the field of bioactive peptides has intensified in the past two decades (for recent reviews, see for instance [1–3]).

The potential of bioactive peptides to improve consumer’s health is a subject of great interest to the scientific community and the food industry. Different chemical and biological methods have been developed and applied to screen their bioactivity, and in some cases, the postulated effects have also been proved in animal and human studies. In addition, the identification and characterization of these peptides is performed using different analytical techniques namely, electrophoresis, chromatography, immunochemical methods, and mass spectrometry in its different modes. A detailed description of these analytical techniques and their application to dairy peptides has been recently reviewed [4]. Research performed to date has found certain structure–activity relationships for bioactive peptides, that is, peptides with a given activity share specific structure cluster or have sequence requirements.

This chapter deals with the methods applied to test the bioactivity of food-derived peptides. The common structural features of peptides exhibiting a particular activity will also be summarized. Special
attention will be focused on the *in vitro* methods used to evaluate bioactivity and the *in vivo* animal models employed.

### 3.2 Evaluation of Peptide Bioactivity

Evaluation of peptide bioactivity is one of the most important challenges in the search for novel bioactive sequences and usually it cannot be accomplished by the use of a single method. To perform an initial screening of numerous sequences or fractions, rapid and simple *in vitro* methods are required. Once they are liberated, they can act locally, at any point of the gastrointestinal tract, or they have to be absorbed to reach the target organ. In this latter case, bioavailability of the peptides and changes that can occur during digestion, absorption, or distribution have to be evaluated using cell cultures, *ex vivo* organ preparations, or *in vivo* animal models. Finally, there is consent over the fact that health claims must be proved in human studies.

#### 3.2.1 Antihypertensive Peptides

Antihypertensive peptides are probably the most extensively studied bioactive peptides from foods and, in fact, an important progress has been made in the area of blood pressure reduction by the use of food peptides. This can be, at least partially, explained because hypertension is a significant problem worldwide and one of the major controllable risk factors associated with cardiovascular diseases [5]. Inhibition of angiotensin-converting enzyme (ACE) is regarded as the main mechanism of action of the antihypertensive activity of food peptides. ACE plays an important role in the renin–angiotensin system, regulating arterial blood pressure, as well as the salt and water balance. ACE hydrolyzes angiotensin I into angiotensin II, a potent vasoconstrictor, whereas it degrades bradykinin, a potent vasodilator [6]. The measurement of the reduction in the formation of angiotensin II *in vitro* is a common test for evaluating ACE inhibition exerted by different drugs and food peptides. The IC$_{50}$ value (the inhibitor concentration that leads to 50% inhibition) is used to compare the effectiveness of different ACE inhibitory peptides. Once the IC$_{50}$ value has been established, it represents an approximation to the possible antihypertensive effect of these compounds [7].

Several methods can measure the ability of food peptides to inhibit ACE *in vitro*. One of the most commonly used ones was developed by Cushman and Cheung (1971) [8], and later modified by Nakamura et al. [9]. This method employs the compound hyppuryl–histidil–leucine (HHL) as the substrate of ACE, which releases the dipeptide His–Leu and hyppuric acid. The amount of hyppuric acid is quantified spectrophotometrically at 228 nm once it has been extracted with ethyl acetate. However, it is a tedious process that can overestimate ACE activity if nonhydrolyzed HHL is also extracted, although this can be avoided by HHL separation and quantitative determination by high-performance liquid chromatography (HPLC) that also has the problem to be a time-consuming method to evaluate ACE-inhibitory activity [10].

Other spectrophotometric methods have been optimized and validated, such as that based on the release of the dipeptide Gly-Gly (GG) when the enzyme reacts with the substrate furanacryloyl-Phe-Gly-Gly (FAPGG) [11]. This method was later evaluated and it was demonstrated that the level of ACE activity in the assay determines apparent IC$_{50}$ value obtained. Therefore, for comparative purposes, it is necessary to detail the number of enzyme units used and include in the study a standard ACE inhibitor, such as Captopril [12]. Later, a fluorescence assay which employs o-aminobenzoylglycyl-p-nitrophenylalanlyproline as the substrate of ACE was developed [13]. The fluorescence generated by the release of the o-aminobenzoylglycyl group is read on a microplate fluorimeter, resulting in a simple, rapid, and sensitive method that allows a continuous monitoring of ACE activity in just one step.

A biochemical assay based on a continuous-flow enzyme–substrate reaction and the subsequent detection of the reaction products by mass spectrometry has been developed and applied to the screening of complex extracts of peptides with ACE-inhibitory activity. The inherent advantage of this method is that the fractionation of peptides for further activity testing is avoided, although an online HPLC-mass spectrometer is needed [14]. Recently, a new turbulent flow cytometry (TFC)/electrospray ionization mass spectrometry (ESI-MS)-based screening method has been developed. In contrast to the methods
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previously described, this approach uses the natural ACE substrate angiotensin I, instead of artificial substrates. The ACE assay is carried out in an atypical offline setup by incubation of the samples with ACE and angiotensin I followed by stopping the reaction with acetonitrile containing val5-angiotensin I, which serves as an internal standard. Angiotensin I and the product angiotensin II are extracted from the incubation mixture by TFC applied in backflush mode as online solid-phase extraction and are directly quantified by ESI-MS. The presence of inhibitors is detected by an increase in the angiotensin I signal intensity and a corresponding decrease of angiotensin II signal, as compared to the blank assay. This parallel (two-way) measurement of the inhibitory activity allows the exclusion of false-positive results caused by interfering matrix compounds. No laborious cleanup is required and the incubation mixture can be analyzed directly after stopping the ACE conversion. The whole TFC/ESI-MS analysis takes <5 min making the method also suitable for a rapid screening of a large set of samples [15].

The structure–activity relationship of ACE-inhibitory peptides has been extensively studied and it is accepted that binding to ACE is strongly influenced by the C-terminal sequence of the peptide. ACE-inhibitory peptides seem to share, as a common structural property, the presence of hydrophobic amino acid residues (with aromatic or branched side chains) at each of the three C-terminal positions. Some of the most potent ACE-inhibitory peptides reported have a proline residue at the end of their sequence. In addition, the presence of a residue of proline at the C-terminal and antepenultimate position appears to enhance binding while ACE only binds weakly to peptides that have penultimate proline residues [16,17]. Later on, it has also been confirmed that the presence of another hydrophobic amino acid, such as leucine as C-terminal end helps inhibit ACE [18]. C-terminal lysine or arginine also seems to contribute to the inhibitory activity of the peptides, which suggests a possible interaction between a positive charge of the inhibitor and an anionic-binding site of ACE [19]. For instance, when the C-terminal proline residue of peptide LHLPLP was substituted by arginine (LHLPLR), the resultant peptide was twice more potent than the parent peptide. However, substitution of the C-terminal proline by leucine yielded a similar activity [20]. It is important to note that ACE-inhibitory activity also depends on the conformation of the peptides. It has been shown that the peptide DKIHP containing \textit{trans}-proline exerts a more potent ACE-inhibitory activity than the conformer with \textit{cis}-proline. It was suggested that the change of a \textit{trans}-form into a \textit{cis}-form of proline could cause significant changes in the peptide structure and in its interaction with the active site of the enzyme [21].

Very often, the attempts to corroborate the antihypertensive effect of peptides with ACE-inhibitory properties reveal a lack of correlation between the \textit{in vitro} ACE-inhibitory activity and the \textit{in vivo} action [22]. This is probably because the selection of potentially antihypertensive food peptides is solely based on the \textit{in vitro} determination of the ACE-inhibitory activity, while important aspects such as the physiological transformations that determine the bioavailability of the peptides or the existence of other mechanisms of action are overlooked [23]. In fact, antihypertensive food peptides with mechanisms of action other than ACE inhibition, such as those possessing antioxidant, vasodilator, and opioid activities, have been reported [24–28].

Some peptides derived from food proteins exert direct effects on vascular smooth muscles [25] or modulate the release of endothelium factors that relax or contract vascular smooth muscles [26,28–32]. To study the vasodilator activity produced by different peptide sequences, organ baths are used. Isolated rings from conduit arteries or resistance vessels from normotensive or hypertensive rats are mounted in isolated tissue chambers containing a physiologic solution at 37°C and continuously bubbled with a 95% O2–5% CO2 mixture, which gives a pH of 7.4. After an equilibration period, the functionality and the maximum contractility of the rings are tested by adding KCl. The functional integrity of the endothelium can be confirmed by the relaxation response to acetylcholine of segments previously contracted with a vasoconstrictor agent. The absence of relaxation induced by acetylcholine confirms the absence of endothelium. Afterward, the relaxation produced by a single (not >0.1 mM) or several concentrations (0.1 µM–0.1 mM) of the peptide can be evaluated in precontracted segments. The effects of different inhibitors such as a nitric oxide (NO) synthase inhibitor (L-NAME), or a cyclooxygenase inhibitor (indomethacin), on the relaxation induced by the peptides can be studied by their addition 30 min before precontraction of the vessels.

There are very few studies dealing with the effects of food peptides on the vascular function, so that the structure–activity relationship of vasodilator peptides is not as yet well established. In contrast
to the ACE-inhibitory activity, it seems that the N-terminal residue is more important than the C-terminal residue to the vascular-relaxing activity [33]. It has been suggested that amino acids, such as arginine or tyrosine, are important at the N-terminal position [28,34]. In addition, small variations in the amino acids at certain positions could lead to different vasorelaxing mechanisms. This is the case of the hypotensive peptides ovokinin (2–7) (RADHPF) and novokinin (RPLKPW). NO and B2 bradykinin receptors are implicated in the mechanism of action of ovokinin [29,31], while the vasorelaxing activity of novokinin, a potent hypotensive peptide acting through the AT$_2$ receptor, is mediated by prostacyclin [35].

In any case, to evaluate the antihypertensive activity of these compounds, experiments on animals and humans are necessary and of particular importance. Several studies have been performed using animal models to determine the antihypertensive effects of food peptides after short- and long-term treatments [22,36–42]. Spontaneously hypertensive rats (SHRs), developed from Wistar rats bred at Kyoto University [43], constitute the most widely used experimental model for hypertension, because the basic principles associated with the onset of hypertension in these animals are surprisingly similar to those in humans [44,45]. Wistar–Kyoto rats are the normotensive control of SHRs. The rats’ arterial blood pressure is usually measured by a modification of the tail cuff method, a noninvasive method originally described by Buñag [46]. In most studies, the acute effect caused by the oral administration by gavage of a single dose of the peptide is evaluated and the rats’ arterial blood pressure is measured before this administration, and then at 2 h intervals for 8 h. Arterial blood pressure is again measured 24 h after administration. For these acute administration experiments, rats with stable arterial blood pressure values (and thus older than 20 weeks) should be used. As an example, Figure 3.1 shows the antihypertensive effect of egg-white-derived peptides after a single oral administration to SHRs (Figure 3.1) [39]. The antihypertensive activity can also be evaluated using radiotelemetric monitoring in conscious rats [47,48]. The telemetric transmitters are implanted into the abdominal aorta by a feasible surgical procedure. This method has the advantage that it avoids the animal stress produced when several measurements are performed in a small period of time.

Hypertension is a chronic pathology which requires chronic treatments; therefore, it is advisable to follow the changes in the arterial blood pressure of SHRs caused by a long-term oral intake of food peptides. In these experiments, the development of hypertension in the rats and the role played by the peptides administered is assessed. In experimental studies of chronic administration to SHRs, arterial

![Graph](image)

**FIGURE 3.1** Decrease in systolic blood pressure (SBP) caused in SHRs by the administration of water (○), captopril (50 mg/kg) (□), and the peptides (10 mg/kg): YRGGLEPINF (●), RDILNQ (▲), and ESIINF (■). The data represent the mean values ± SEM for a minimum of five animals. *P < 0.05 versus water; #P < 0.05 versus captopril; $P$ was estimated by a two-way analysis of variance. (From M. Miguel et al. *J Agric Food Chem.* 55: 10615–10621, 2007. With permission.)
blood pressure is measured at the same time of day (usually in the morning), in order to avoid the influence of the circadian cycle. In these studies, the treatment begins at weaning and the products are usually administered in the animals’ drinking water. Continuation of the measurements after withdrawal of the treatment allows the evaluation of the possible reversal of the effects. Food intake, water intake, and body weight gain in the animals can be recorded weekly during all experimental period. At the end of the study, blood and tissue samples of the rats can be obtained after an overnight fasting period, in order to perform biochemical studies to explore the mechanisms of action implicated in the antihypertensive effect of the product.

There are several differences in the bowel structure, function, and microflora between rodents and humans. It is therefore evident that it is necessary to carry out clinical studies in humans to demonstrate the efficiency of the antihypertensive peptides and to guarantee their security. However, only a few human clinical studies have been carried out [49–59]. Recent data do not seem to support a positive role of bioactive peptides in blood pressure regulation. However, beneficial effects in hypertensive subjects from specific populations cannot be discarded, and more research is needed to elucidate mechanisms other than the intervention in the renin–angiotensin system that could be involved [60].

The results of the above-mentioned studies suggest the possibility of using hydrolyzates and peptides from food proteins as health-enhancing ingredients in functional foods, nutraceuticals, and pharmaceutical preparations to reduce the risk of hypertension. Their usefulness may be particularly clear in prehypertensive subjects who do not need antihypertensive medication yet, and who control their blood pressure by dietary means. It is also likely that these products can be of use in hypertensive patients who do not respond well to pharmacological treatments. It should be noted that several of these peptides, which have already proved their safety and effectiveness in hypertensive patients, are currently marketed in functional foods [61].

### 3.2.2 Antioxidant Peptides

Oxidative stress caused by an abnormal production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in cells has been implicated in the etiology of age-associated chronic diseases, such as cardiovascular diseases, cancer, diabetes, neurodegenerative disorders, and aging [62]. Moreover, it is well known that lipid peroxidation occurring in food products causes deteriorations of food quality and shortening of shelf life. In the past years, the importance of antioxidants in the protection of organisms, tissues, and nonliving systems against oxidative stress has become evident. Artificial antioxidants (butylated hydroxyanisole, butylated hydroxytoluene, and \(n\)-propyl gallate) exhibit a strong antioxidant activity against several oxidation systems. However, there is growing concern about their safety, particularly their potential carcinogenicity and genotoxicity [63,64]. This, along with consumer preference for natural occurring bioactive components, has prompted researchers to identify and develop novel natural and cost-effective antioxidants. Recent studies have suggested that food proteins hold promise as a potential dietary source of natural antioxidants. Hydrolyzed proteins from many animal and plant sources, such as milk [65,66], soy [67,68], zein [69], rice bran [70], canola [71], egg-white and yolk [24,72], porcine myofibrillar [73], and fish proteins [74–79] have been found to possess antioxidant activity. This activity is believed to be attributed to peptide sequences contained in these proteins and released after enzymatic proteolysis.

The ability of peptide fractions to inhibit deleterious changes induced by lipid oxidation appears to be related to their nature and composition. This seems to be dependent on the specificity of the proteinase used to generate the hydrolyzate [80]. There is evidence that the antioxidant effect of individual amino acids increases when they are incorporated in a peptide, indicating that the peptide bond or the structural conformation have an influence of this activity [81]. Thus, the peptide composition can lead to both synergistic and antagonistic effects with regard to the antioxidant activity of free amino acids. Chen et al. (1996) found that peptides containing a Pro-His-His sequence exhibited the greatest antioxidant activity among a group of tested peptides [67]. In addition, high levels of histidine and of some hydrophobic amino acids were related to peptide antioxidant potency [82]. The activity of histidine-containing peptides is thought to be connected to their hydrogen-donating ability, lipid peroxy-radical trapping, and/or the metal-ion chelating ability of the imidazole group [83]. Other amino acids such as tryptophane and
tyrosine have also been reported to contribute to the antioxidant activity, because of the capacity of the indolic and phenolic groups, respectively, to serve as hydrogen donors [24,66]. The hydrophobicity of the peptide also appears to be an important factor for its antioxidant activity due to increased accessibility to hydrophobic targets (e.g., lipophilic fatty acids) [84].

Generally, the ideal method for determination of antioxidant properties of peptides should assess their effect under reaction conditions that mimic those occurring when oxidative stress is induced in vivo by RNS and ROS [85]. As it has been observed for other antioxidants, the activity of peptides depends on the assay considered. The antioxidant properties of peptides have been attributed to the cooperative effect of a number of properties, including their ability to scavenge free radicals, to act as metal-ion chelators, oxygen quenchers, or hydrogen donors, and to the possibility of preventing the penetration of lipid oxidation initiators by forming a membrane around oil droplets. According to these properties, diverse methods have been proposed to measure the antioxidant activity of peptides derived from food proteins.

In the last few years, the Oxygen Radical Absorbance Capacity assay has been widely used to determine the antioxidant capacity of peptides derived from milk-whey proteins [66,81,86], egg [24], soy [87], seafood muscle [88], and fish proteins [89]. In the basic assay, the peroxyl radicals generated react with a fluorescent oxidizable protein substrate to form a nonfluorescent product. Probe reaction with peroxyl radicals is followed by a loss of the intensity of fluorescence with time. This assay measures the antioxidant inhibition of peroxyl-radical-induced oxidations and reflects a classical radical chain breaking antioxidant activity by H-atom transfer [90]. The low cost of the material and the possibility of using an automated fluorescence reader are the two main advantages of this assay. Moreover, it is particularly useful for samples which contain multiple ingredients with antioxidant activity.

The Trolox Equivalent Antioxidant Capacity (TEAC) assay has become one of the most widely used methods in testing the antioxidant capacity of food peptides from different sources. It has been applied to vegetal proteins, such as soy [91,92], zein [93], and rice bran proteins [94], among others. Hydrolyzates from animal proteins, such as milk and fish proteins, have also been analyzed for antioxidant activity with this method [66,95–97]. This assay uses 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), a peroxidase substrate, which when oxidized, generates a metastable radical (ABTS•+). This radical is intensely colored and can be monitored spectrophotometrically in the range of 600–700 nm. The antioxidant capacity is measured as the capacity of test compounds to decrease the color by reacting directly with ABTS•+ radical, and expressed relative to that of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) [98]. This assay is often used to rank antioxidants to study structure–activity relationships [99]. Because the ABTS•+ radical can be solubilized in both aqueous and organic media and it is not affected by the ionic strength, the antioxidant capacity of hydrophilic and lipophilic compounds can be measured [100]. However, this method has two important limitations. On the one hand, the TEAC value actually characterizes the capability of the tested sample to react with ABTS•+ rather than to inhibit the oxidative process. On the other, the ABTS radical used in TEAC assays is not similar to the radicals found in biological systems [101].

In the 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Capacity Assay, the purple chromogenic radical DPPH is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine. The reducing ability of the antioxidants can be evaluated by monitoring the absorbance decrease at 515–528 nm, until the absorbance remains stable or by electron spin resonance. This assay is technically simple and rapid and it only requires a UV–vis spectrophotometer that might explain its widespread use in antioxidant screening. An important limitation of this method is the interpretation of the role of hydrophilic antioxidants, because DPPH can only be dissolved in organic media [100]. However, many authors have used this assay for measuring the antioxidant activity of peptides from food proteins [87,93,102–108].

The ferric reducing antioxidant power assay is based on the reduction of yellow ferric tripyridyltriazine complex (Fe(III)-TPTZ) to blue ferrous complex (Fe(II)-TPTZ) by the action of electron-donating antioxidants [109]. The resulting blue color, measured spectrophotometrically at 593 nm, is taken as linearly related to the total reducing capacity of electron-donating antioxidants. This assay is rapid, simple, robust, inexpensive, and it does not require specialized equipment. Therefore, it has become one of the preferred methods to determine the antioxidant activity of peptides from several food protein sources [88,96,110].
In general, *in vitro* low-density lipoproteins (LDL) oxidation has been effectively used in several studies to characterize the antioxidant capacity of a number of different phytochemicals [111–114] and food peptides [115–117]. This method artificially induces the autoxidation of linoleic acid or of LDL particles, and the peroxidation of the lipid components is determined through the formation of conjugated dienes, followed by a spectrophotometrical analysis at 234 nm [118]. Basically, there are three mechanisms involved: free radical scavenging activity, binding to critical sites on LDL, and metal chelation [112]. Currently, different methods are being used to analyze the inhibitory properties of food peptides on ROS generation and on lipid peroxidation in different cell lines as the basis of their ability to reduce oxidative stress. For instance, estrogen-independent breast cancer cells were employed by Hernández-Ledesma et al. [92] to evaluate the antioxidative effect of the peptide lunasin in macrophages RAW 264.7.

Many food-derived peptides are effective as *in vitro* antioxidants but, due to the variable reports on their *in vivo* efficacy and the pro-oxidant nature of some antioxidants, it is necessary to perform studies in experimental animal models fed with these components to demonstrate the reduction of *in vivo* oxidative stress by the use of different biomarkers. It is known that compounds that upregulate the production of endogenous antioxidants, such as glutathione and antioxidant enzymes, provide novel approaches for the restoration of redox homeostasis. The values and enzymatic activities of these compounds, as well as the total antioxidant capacity, can be evaluated in the plasma and tissues of animals by enzymatic–colorimetric methods [119–121].

### 3.2.3 Antimicrobial Peptides

In the past 20 years, there has been an increasing interest in antimicrobial peptides present in men and animals, because they are considered to possess the functions in host defense, inflammation, and tissue generation [122,123]. In addition to these, it has also been demonstrated that several food proteins and peptides exert antimicrobial activities that can also provide protection against infection [124,125].

Numerous studies have been performed to understand the relationship between the structure and the activity of antibacterial peptides, especially of antibiotics peptides that act as components of the host defenses of animals or plants. An amphiphilic, mostly \( \alpha \)-helical conformation, and an excess of positive charges are recognized as major structural motifs determining the membrane-disturbing activity of these peptides. These cationic and hydrophobic peptides are demonstrated to act by direct interaction with specific anionic membrane components, orientated toward the exterior of the cell, promoting pore formation and bacterial membrane disruption. Other peptides have membrane-permeabilizing properties and cell-penetrating activity. Some of these structural characteristics are also found in food-derived peptides.

The antibacterial activity of some dietary peptides can be tested by an agar diffusion assay where the peptide diffuses from a paper disc or small cylinder into an agar medium that contains test organisms. Inhibition is observed as the failure of the organism to grow in the region of the peptide. This method has been used, for instance, as a preliminary test to characterize the antibacterial activity of peptides obtained after the fermentation of casein with *Lactobacillus acidophilus* [126] and of peptides from human milk [127]. More frequently, the antimicrobial activity is determined in a liquid medium, commonly by using a microtiter plate assay, and measuring bacterial growth by optical density or bacterial count. Results are compared by establishing the minimum inhibitory concentration of a peptide/hydrolyzate for a given microorganism, that is, the lowest peptide/protein concentration that shows no growth at the end of the experiment. To compare the activity of different peptides or hydrolyzates, the ICs\(_{50}\) value, defined as the concentration required to obtain 50% inhibition of growth at the exponential phase of growth, is also employed [128]. This assay has been widely used and recent applications include the assay of the antimicrobial activity of human milk against *Escherichia coli* and *Listeria monocytogenes* [129], synthetic porcine lactoferricin against *Staphylococcus aureus* and *Candida albicans* [130], \( \alpha_{s2} \)-casein-derived peptides [131], and lactoferramind [132].

The use of a bioluminescence assay to follow bacterial growth and metabolism has been also applied to investigate the effect of hydrolyzates of \( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin against *E. coli* JM103 [133]. Bacterial enumeration through fluorescent *in situ* hybridization has also been used to study the effect of \( \alpha \)-lactalbumin and glycomacropeptide on mixed populations of human gut bacteria [134]. In the
latter example, in addition to a batch culture, a two-stage continuous culture model was used, with the advantage of allowing spatial and temporal heterogeneity and the assay of more complex environmental conditions.

Electron microscopic examination of bacterial preparations has also been used for lactoferricin, other lactoferrin-derived peptides [135], and \( \alpha_s2 \)-casein-derived peptides [129]. As an example, Figure 3.2 shows the transmission electron microphotographs corresponding to intact cells of \( E. \ coli \) ATCC 25922 cells and cells incubated with bovine \( \alpha_s2 \)-casein \( f(183–207) \) or lactoferricin. This technique allows the detection of damage in bacterial membranes and changes in the cytoplasm. With the aim of studying the mechanism of action of these antibacterial peptides, other techniques, such as circular dichroism analysis with micelles or liposomes, peptide binding to microbial cell components, and nuclear magnetic resonance spectroscopy, among others, have been successfully employed, but they fall out of the scope of this chapter.

There exist only a few studies on the \textit{in vivo} activity of antimicrobial dietary peptides performed using animal models or in clinical trials in humans. Studies using animal models have reported a protective effect of orally administered lactoferricin against infections by methicillin-resistant \( S. \ aureus \) [136] or by the parasite \( T. \ gondii \) [137]. Most of these studies were performed to demonstrate the antibacterial effects of the entire protein lactoferrin after oral administration. However, as orally administered lactoferrin is partially degraded to fragments that contain the lactoferricin sequence [138,139], some of these effects can probably be attributed either to lactoferrin fragments or to the combined action of lactoferrin and its derived peptides.

In humans, a study with low birth-weight infants fed with a lactoferrin-enriched infant formula concluded that lactoferrin contributes to the formation of a bifidobacteria-rich flora [140]. Lactoferrin has also demonstrated to be effective in the eradication of \( H. \ pylori \) when used as a supplement.

**FIGURE 3.2**  TEM micrographs of \( E. \ coli \) ATCC 25922. (a, b) Untreated cells. The arrow in (b) indicates the periplasmic space. (c, d) Cells treated with bovine \( \alpha_s2 \)-casein \( f(183–207) \) at a concentration of 31 \( \mu \)M during 2 h at 37°C. Arrows in (d) indicate the loss of continuity of the membrane and the swollen area between inner and outer membranes. (e, f) Cells treated with lactoferricin-B at a concentration of 31 \( \mu \)M during 2 h at 37°C. The arrow in (e) indicates a blister in the outer membrane. (From I. López-Expósito, L. Amigo, and I. Recio. \textit{BBA Biomembranes} 1778: 2444, 2008. With permission.)
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to an antibiotic treatment [141]. Other in vivo studies on lactoferrin to investigate its antiviral and immunomodulatory effects, as well as other host-protective activities, such as cancer prevention, and clinical applications have been reviewed [142,143].

There exist some studies on the in vivo activity of isracidin, corresponding to αs1-casein f(1–23). In mice, it exerts a protective effect against L. monocytogenes, Streptococcus pyogenes, and S. aureus. Protection of rabbits, guinea pigs, and sheep against S. aureus has also been achieved. In cows with mastitis, isracidin obtained a success rate of over 80% in the treatment of chronic streptococcal infection. Furthermore, it has been demonstrated that isracidin possesses immunomodulating properties, with a significant effect in the production of IgG, IgM, and antibody-forming cells and an enhancing effect on cell-mediated immunity when injected to mice [144]. Another interesting finding was made with a tryptic casein hydrolyzate for the treatment and prophylaxis of newborn calf colibacillosis [145]. Recently, a product obtained from bovine colostrum rich in immunoglobulins, growth factors, antibacterial peptides, and nutrients was demonstrated to reduce the number of evacuations of stools per day in patients with HIV-associated diarrhea [146].

3.2.4 Opioid Peptides

Accumulated evidence shows that peptides from food proteins may act as opioid receptor ligands, and thus, they may be regarded as exogenous supplements to the endogenous opioidergic systems of the human organism. They have been referred to as exorphins, to distinguish them from opioid peptides of endogenous origin, called endorphins. Opioid receptors (μ-, δ-, and κ-type) are located in the nervous, endocrine, and immune systems, as well as in the intestinal tract of the mammalian organism and they can interact with their endogenous ligands, as well as with the dietary opioid and opioid antagonists [147]. Orally administered food-protein-derived peptides have demonstrated to influence postprandial metabolism by stimulating the secretion of insulin and somatostatin [148,149], to modulate intestinal transport of amino acids [150], to prolong gastrointestinal transit time, and to exert antidiarrheal action [151]. Recently, it has been proposed that β-casomorphin-7 may contribute to mucin production via a direct effect on the intestinal goblet cells and an activation of μ-opioid receptors [152]. They may also play a role in the reproductive function of the female organism, supporting processes of pregnancy, delivery, and lactation period, while they may exert a cardioprotective function in pregnant or lactating mammals [153].

The common structural characteristic of opioid peptides is the presence of a tyrosine residue at the amino terminal end (except milk-derived α-casomorphins that have an additional arginine residue) and the presence of another aromatic residue in the third or fourth position. The negative potential, localized in the vicinity of the phenolic hydroxyl group of tyrosine, is essential for opioid activity and the presence of a proline residue helps maintain the proper orientation of the tyrosine residue and the other aromatic amino acids [154,155]. The occurrence of aliphatic amino acids in the third position also contributes to the opioid activity in the case of gluten exorphins and neocasomorphin (YPVEPF).

Food-derived peptides can be screened for opioid activity by measuring the adenylate cyclase activity in homogenates of neuroblastoma X-glioma cells and using naloxone as opioid antagonist to reverse the effect [156]. The two bioassays employed more often to test materials with opioid activity are the guinea-pig ileum longitudinal muscle myenteric plexus preparation and the mouse vas deferens preparation. Opioid receptors located on intestinal smooth muscle cells are the targets for exogenous μ- and κ-opioid receptors agonists to inhibit the propulsive peristaltic activity and to increase the smooth muscle tone [157]. The mouse vas deferens are classical preparations for studying the pharmacology of δ-opioid receptors [158]. The activity in these two organ bath preparations can be antagonized with opioid antagonists, such as naloxone. Further, specific radioligand competition assays for opioid μ-, δ-, and κ-sites have also been used for assessment of opioid peptides [159].

Several animal models have been used after oral or intragastrointestinal administration of food-protein-derived opioid peptides, which allowed the demonstration of a number of activities for β-casomorphins, milk casein preparations, gluten exorphins, and gluten or gluten hydrolyzates. In dogs, stimulation of insulin release, reduced gastrointestinal motility, and release of somatostatin are observed after intragastric administration of casein [148,160,161]. In rats, satiety was demonstrated to be influenced by casein or casein hydrolyzate [162]. Prolongation of gastric emptying rate and gastrointestinal transit time are
also observed after intragastric administration of casein [151]. Several studies have also been performed by using calves and cows. For instance, an antidiarrheic effect is found in calves upon oral administration of β-casomorphin(1–4) amide [163]. The behavioral and antinociceptive effects of these peptides are usually tested in mice. The tail pinch method has been employed in the case of a peptide derived from Rubisco [164]. Effects on nociception, locomotor activity, motor coordination, rectal temperature, and duration of pentobarbital anesthesia have been evaluated in mice after intraperitoneal administration of α-lactorphin (YGLF, an opioid tetrapeptide derived from α-lactalbumin). This peptide, in contrast to morphine, elicits no behavioral effect in mice [165]. Interestingly, this α-lactalbumin-derived peptide has the ability to lower blood pressure in hypertensive and normotensive rats after subcutaneous administration [47]. Another αs1-casein-derived opioid peptide, RYLGY, has recently shown antihypertensive activity in SHRs after acute and chronic administration [27,166].

In adult humans, oral administration of β-casomorphin(1–4) amide delays the gastrointestinal transit time [167]. A gluten hydrolyzate also causes a prolongation of gastrointestinal transit time in healthy volunteers, an effect that is blocked by naloxone [168].

### 3.2.5 Mineral-Binding Peptides

The first reference to bioactive peptides in the scientific literature was made by Mellander in 1950, who suggested that casein-derived phosphorylated peptides enhanced vitamin D-independent bone calcification in rachitic infants [169]. Later, it was demonstrated that these peptides, also named phosphopeptides, by virtue of their high content of negative charges, efficiently bound divalent cations, specially calcium, but also Fe, Mg, Mn, Cu, and Se, forming soluble complexes. It has also been suggested that the mineral-binding activity could be dependent on the histidine content and peptide size [170,171]. These peptides with mineral-binding activity could then function as carriers of various minerals and thus, either enhance or inhibit their bioavailability.

Mineral-binding peptides are considered physiologically beneficial in the prevention of dental caries, mainly because of their role in recalcification of the dental enamel. The use of these peptides in preventing osteoporosis, hypertension, and anemia has also been proposed [172]. Another interesting property associated with phosphopeptides is their potential to enhance mucosal immunity [173]. It is important to note that the high content of negative charges makes these peptides resistant to further hydrolysis [174], and they have been detected in the gastrointestinal tract of animals and humans after oral ingestion [175–177].

Isolation and characterization of peptides with mineral-binding activity requires selective solubilization and precipitation methods [178–184]. Differences in mineral affinity, competition for binding sites, and changes in the pH and ionic strength of the solvent are used to influence mineral binding and release. Mineral-binding peptides can be also isolated using ultrafiltration and hydroxyapatite affinity (HA) chromatography [185–187], or measuring the calcium content of the supernatant after the formation of a Ca–peptide complex using CaCl₂ and sodium phosphate buffer [188].

The beneficial properties of phosphopeptides can be estimated in vitro by selective precipitation, using a model system based on HA. In this method, the peptides are incubated with a suspension of HA in Tris buffer at pH 7.0, as a replacement for dental enamel and sodium acetate buffer at pH 4.2, is added to simulate the organic acids found in the mouth. After centrifugation, the supernatant is used to measure the levels of calcium and phosphorus dissolved from the HA by the action of the sodium acetate buffer, which represents 100% demineralization. The effectiveness of peptides samples to reduce the demineralization of HA is deduced by comparing calcium and phosphorous concentrations in the protein/peptide supernatant with the values of the controls [189–191].

The measurement of the mineral content is complex and depends on several factors. Among these, the equilibrium status, redox form, solubility, and binding affinity are regarded as some of the most important ones. Sensitivity of the method is also important [174]. For instance, analysis of total calcium, iron, zinc, magnesium, and some trace elements can be performed by atomic absorption spectroscopy [192], colorimetric methods measure the ferrous form of iron [193,194], and chromatography in the presence of chelating agents can be applied as a selective technique [193,195,196]. It is also possible to use radioactive isotopes to follow the binding mechanism [197–199].
Improved *in vitro* methods utilize human colon carcinoma cell lines (Caco-2) [200] to mimic and estimate the uptake and/or transport of mineral elements [201]. Other cell lines can also be employed to assess the potential of peptides to enhance the dietary mineral bioavailability and to modulate the bone formation [202], such as bone-marrow-derived osteoblast precursor cells, to measure the ability of food-derived peptides to stimulate the proliferation of osteoblasts to form osteoclasts [203]. However, these *in vitro* methods only estimate the fraction of elements available for absorption [204], and thus, mineral bioavailability should be evaluated *in vivo* [205], assessing intestinal calcium absorption in rats [206], or enhanced bone calcification [207,208].

### 3.2.6 Antithrombotic Peptides

It is generally accepted that platelet adhesion and aggregation play an important role in the pathogenesis of thrombosis, particularly arteriothrombosis [209]. Antithrombotic agents are widely used in medicine for the treatment of several cardiovascular events and related diseases, such as coronary angioplasts, coronary thromboembolisms, myocardial heart attack, pulmonary embolism, and so on [210]. The formation of thrombin consists of the binding of fibrinogen to its platelet receptors after activation by physiological agonists such as adenosine diphosphate (ADP), thrombin, and collagen [211]. One of the sites at which fibrinogen binds to platelet receptors corresponds to the C-terminal sequence of the γ-chain, the 400–411 fragment (HHLGGAKQAGDV) [212]. *In vitro* and *in vivo* experiments have demonstrated that food peptides analog to this sequence, such as casein macropeptide that derives from κ-casein, are capable of inhibiting the binding of the human fibrinogen γ-chain to fibrinogen receptors on the platelets surface, what would otherwise lead to platelet aggregation [213–218]. These peptides have been detected in plasma of new born children after ingestion of breast milk or infant formula, although their *in vivo* significance remains unclear [219].

The antithrombotic activity can be evaluated *in vitro*, by assessing the inhibition of fibrinogen binding to ADP-activated platelets. For this purpose, platelet-rich plasma is incubated with the peptides and, after addition of ADP, the increase in light transmission is monitored using an aggregometer [175,213,220]. The antithrombotic activity can be also studied *in vivo* using a guinea-pig model of arteriolar thrombosis, which is triggered by a laser-induced minimal injury [216,221–223].

### 3.2.7 Hypocholesterolemic Peptides

Hyperlipidemia, especially hypercholesterolemia, is one of the most important risk factors contributing to the development of cardiovascular diseases [224–227]. Plasma cholesterol levels are influenced by the diet and by cholesterol biosynthesis, uptake, and secretion [228–231]. Considerable evidence indicates that cholesterol lowering stabilizes atheroma plaques reducing cardiovascular events [232–235]. In the last few years, numerous drugs and natural extracts have been explored for their potential in the prevention and treatment of hypercholesterolemia. In this context, food-derived peptides might also lower serum cholesterol levels [229,230,236–239], by increasing cholesterol catabolism, reducing intestinal absorption of dietary cholesterol or inhibiting cholesterol uptake from the plasma into the liver, preventing the deposition of cholesterol into plaques on the arterial wall [240].

Hydrophobicity seems to be responsible for the hypocholesterolemic potential of peptides, especially for their bile acid-binding ability and it has also been suggested that the hypocholesterolemic activity is strongly influenced by the presence of a leucine residue at the N-terminal side [241]. Further work is required to identify hypocholesterolemic peptides, and to clarify the influence of peptide structure on the hypocholesterolemic activity.

Cholesterol is water insoluble and requires a micellar solubilization step prior to intestinal absorption [242]. *In vitro* assays relate the hypocholesterolemic action of proteins and peptides with their ability to decrease the micellar solubility of cholesterol and with their taurocholate-binding capacity. To study micellar solubility *in vitro*, the sample is incubated with either artificially prepared or natural micellar solutions containing sodium taurocholate, cholesterol, oleic acid, and sodium phosphate at pH 7.4 and, after ultracentrifugation, the cholesterol content is measured [236,239]. On the other hand, the taurocholate-binding capacity determines the ability of the peptide to bind bile acids *in vitro* [243,244].
Peptides are incubated with a Tris–HCl buffer containing taurocholate, at 37°C for 30 min, and the content of taurocholate is measured enzymatically after dialysis of the mixture [245,246]. Other in vitro methods apply monolayers of Caco-2 cells as a model system to examine in vitro cholesterol absorption [231,236,247].

In vivo hypocholesterolemic activity can be studied using different animal models, such as rabbits [248], normal rats [249], diabetic rats [250], diabetic and nondiabetic hamsters, [251], guinea pigs [252], and monkeys [253]. For the induction of hypercholesterolemia, they are fed with a cholesterol-enriched diet, prepared by adding cholesterol and cholic acid to the standard diet, during several weeks [254]. Cholic acid is included because it increases micelle formation and facilitates intestinal absorption of cholesterol. At the end of the experimental study, the serum lipid profile (cholesterol and triglyceride levels) can be determined using commercial kits. It is also useful to measure liver function enzymes, such as the rate-limiting enzyme responsible for bile acid biosynthesis, cholesterol 7α-hydroxylase, and the rate-limiting enzyme responsible for the cholesterol synthesis system, 3-hydroxy-3-methylglutaryl coenzyme A reductase [255,256], or the incorporation of [3H]-cholesterol into the serum, liver, and intestine of the animals [257].

3.2.8 Antiproliferative and Cytotoxic Peptides

Cancer has become one of the most common causes of death in industrialized countries and it has been defined as the medical challenge of our times. An impressive body of evidence supports the notion that prevention can be a major component of cancer control. Epidemiological cell culture and animal studies have demonstrated that a large number of natural compounds present in the diet could lower cancer risk and even sensitizes tumor cells in anticancer therapies [258].

Carcinogenesis is a multistage process with an accumulation of genetic alterations, so that the sequence of events has many phases for intervention with the aim of preventing, slowing down, or reversing the process. Based on various cytochemical studies, there is an increasing evidence for the possible involvement of food-protein-derived peptides as specific signals that can inhibit the viability of cancer cells [259]. These studies are based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described by Holst and Oredsson [260]. In this assay, MTT is added to the growth medium of the cells, taken up by living cells via endocytosis and reduced to blue formazan crystals inside the cell via complex I in the mitochondrial respiratory chain. The amount of formazan generated is assumed to be directly proportional to the cell number when using homogeneous cell populations [260]. As an example, this method has been used to evaluate the cell proliferation inhibitory properties of peptides from different food protein sources, such as the lactoferricin [261], lunasin from soy protein [262], and lunatusin from lima beans protein [263].

Induction of apoptosis is considered to be one of the important targets in a cancer preventive approach. A regulated form of cell death is a complex process that involves the active participation of the affected cells in a self-destruction cascade and it is defined by a set of characteristic morphological hallmarks, including membrane blebbing, shrinkage of cell and nuclear volume, chromatin condensation, and nuclear DNA fragmentation due to endonuclease activation. Several studies have demonstrated the role of food peptides as apoptosis-inducing agents in different human cancer cell lines [264,265]. As an example, Hsieh et al. [262] have studied recently the effect of the peptide lunasin, alone or in combination with aspirin on the programmed death of estrogen-independent MDA-MB-231 breast cancer cells (Figure 3.3).

New technologies, such as DNA microarrays and serial analyses of gene expression, have created an exciting new frontier for the nutrition and healthcare community. These technologies allow the simultaneous analysis of the expression of thousands of genes and the comparison between normal and abnormal tissues. Therefore, they are being widely used to identify and characterize the response to preventive and therapeutic interventions. Moreover, these techniques are useful for a better understanding of the mechanisms involved in the cancer-preventive actions of food compounds [262,266]. Other techniques are being developed and refined to assist in the identification of the proteome, defined as all proteins present in the cell in a particular cellular time [267]. This type of investigation is very important because of the fact that gene expression does not always correlate with protein expression and the influence of food components may be either translational or posttranslational, rather than at the transcriptional level [268]. Thus, proteomic-based studies, although technically challenging, complement genomic studies,
Lunasin promotes aspirin-induced apoptosis. (a) Flow cytometry-based Annexin V labeling of apoptotic cells. Cells were treated for 48 h with vehicle, 1 μM lunasin, 10 μM lunasin, 2 mM aspirin, 1 μM lunasin + 2 mM aspirin, and 10 μM lunasin + 2 mM aspirin. (b) Early and late stages of apoptosis were identified by Annexin V+/7-AAD− and Annexin V+/7-AAD+, respectively in breast cancer cells treated with aspirin, lunasin, and their combination at different concentrations. Three independent experiments were performed (*P < 0.0001 versus vehicle-treated cells; # P < 0.05, § P < 0.0001 versus aspirin-treated cells). (From C.-C. Hsieh, B. Hernández-Ledesma, and B.O. de Lumen. Food Chem 125: 630, 2011. With permission.)
being essential in any comprehensive research strategy aimed at examining the molecular processes and factors involved in modulating cancer risk.

The animal experiments represent the next step to demonstrate the cancer-preventive activity of food peptides. Transgenic and knockout models present evidence that genes markedly influence the response to dietary components, also providing valuable clues about their site(s) of action [269–271]. Other animal models commonly used to evaluate the preventive role of food peptides are those based on chemical and radiation-induced carcinogenesis. As an example, the soybean Bowman–Birk protease inhibitor (BBI) and the BBI concentrate have shown to exert a suppressive effect on 7,12-dimethylbenz(a)anthracene-induced oral cancer [272], 3-methylcholanthrene-induced lung cancer [273], methylbenzylnitrosamine-induced esophageal cancer [274], radiation-induced lymphosarcomas [275], and ovarian sarcomas [276]. BBI and other peptides, such as lunasin, have also demonstrated antitumorogenic properties against different types of cancer using xenograft mouse models [277–279]. These models are based on the injection of human tumor cells to evaluate the progress of these cells under the effects of chemopreventive peptides. Finally, some peptides, such as BBI, are currently being used in large-scale human trials, which represent the last step in demonstrating their cancer preventive role [280].

3.3 Future Prospects

The known bioactive peptides present in food, or produced upon food processing or ingestion, can improve the health status through reductions in blood pressure, enhancement of human defenses (antimicrobial, antioxidant, and cytomodulatory peptides), or modulation of the absorption of nutrients (mineral and cholesterol absorption). Future research in the field of bioactive peptides will focus on the identification of novel bioactive sequences, on the possible mechanisms of action and on new health benefits. In addition to the search for novel peptide sequences with traditional functionalities, other bioactivities will be explored. Of special interest are bioactivities related with the gastrointestinal tract. For instance, it has been found that peptides can affect gastric hormones that control gastric and intestinal motility and secretions or stimulate the release of satiety hormones, such as cholecystokinin [281,282]. Some of these activities are exerted by modulating and influencing gene expression, being the role of dietary peptides in nutrigenomics an undoubtedly growing field of research [283].

The techniques used to study such benefits have improved over the recent years and unique tools have now become available that facilitate the undertaking of challenges that were impossible only one decade ago. The use of advanced peptidomic analytical tools, mainly tandem mass spectrometry analysis, has led to the identification of minor peptides in complex mixtures and biological fluids. Theoretical predictions and simulations are also considered emerging tools in peptide science. These include structure–activity studies based on in silico analysis using chemometric methods and the use of computational chemistry for the creation of sequence databases of bioactive peptides.

New genomic, proteomic, and metabolomic techniques are now enabling the finding of novel associations between diet and chronic diseases through examination of the functional interactions of food components with the genome at molecular, cellular, and systemic levels. Advances in this area will allow the identification of new biomarkers of the physiological effects of peptides that can be employed to evaluate the peptide bioactivity. The mechanisms underlying many of the biological properties of food-derived peptides are, and will be, the subject of increasing interest. Evidence for the beneficial effects of these peptides and demonstration of their mechanisms of action should be conclusive for the approval of a health claim by the authorities. Although some bioactive sequences are already in the market, it is expected that these peptides will be optimally exploited for human nutrition and health when knowledge in these different areas increases.

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